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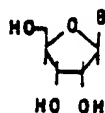
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DOC

(54) A method for preparing a mononucleotide-3'-phosphodiester-based substrate.

(57) A method for preparing a substrate capable of undergoing catalytic-induced hydrolysis of the phosphate ester at the 3'-position to yield a species capable of being monitored spectrophotometrically or fluorometrically, comprises  
(a) blocking a mononucleoside of the formula:



wherein B is a nucleotide base, and wherein the CH<sub>2</sub>OH group at the 4'-position is either *cis* or *trans* to said base, with a silyl blocking member at both the 2'- and 5'-hydroxyls of said mononucleoside to form a 2', 5'-diblocked mononucleoside; and  
(b) forming a 2', 5'-diblocked mononucleotide 3'-phosphodiester by bonding said 2', 5'-diblocked mononucleoside with a moiety selected from the group consisting of a chromophore or fluorophore;  
said silyl blocking member at the 2'-hydroxyl being capable of at least essentially blocking medium-induced hydrolysis of the phosphodiester at the 3'-position, and said silyl blocking member at least at the 2'-hydroxyl being capable of being removed to provide a substrate characterized by the ability to undergo catalytic-induced hydrolysis of the phosphate ester at the 3'-position to yield a species capable of being monitored spectrophotometrically or fluorometrically. The silyl blocking member at least at the 2'-hydroxyl is removed so as to provide a substrate characterized by the ability to undergo catalytic induced hydrolysis of said phosphodiester to yield a species capable of being monitored spectrophotometrically or fluorometrically.

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wherein B is a nucleotide base, and wherein the CH<sub>2</sub>OH group at the 4'-position is either *cis* or *trans* to said base, with a silyl blocking member at both the 2'- and 5'-hydroxyls of said mononucleoside to form a 2', 5'-diblocked mononucleoside; and

(b) forming a 2', 5'-diblocked mononucleotide 3'-phosphodiester by bonding said 2', 5'-diblocked mononucleoside with a moiety selected from the group consisting of a chromophore or fluorophore;

said silyl blocking member at the 2'-hydroxyl being capable of at least essentially blocking medium-induced hydrolysis of the phosphodiester at the 3'-position, and said silyl blocking member at least at the 2'-hydroxyl being capable of being removed to provide a substrate characterized

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A Method for Preparing a Mononucleotide-3'-phosphodiester-based Substrate

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to chromogenic and/or fluorogenic mononucleotide 3'-phosphodiesters, and, more particularly, to a novel method for synthesizing such mononucleotide phosphodiesters. These materials may be used, for example, in carrying out various non-isotopic immunoassays.

2. Description of the Prior Art

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For a variety of clinical purposes such as, for example, monitoring dosage schedules, monitoring hormone levels, checking for recent ingestion or following pharmacological dynamics of bioavailability, absorption, degradation or excretion, it is a great advantage to measure the concentration of various drugs or the like to the nanomolar or even picomolar level. As is known, radioimmunoassay can accomplish analyses of this type. To carry out an analysis, an acceptable kit or system must include an antiserum, a standard of the compound (i.e., - analyte) to be measured, the radiolabeled derivative of the compound to be measured, a buffering agent or agents and, often, a displacing agent. The antiserum is produced by bleeding animals which have been immunized by inoculation, for example, with the hapten - protein conjugate (immunogen) corresponding to the compound to be measured.

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As is well known, in general, the technique of radioimmunoassay measures the competition between radioactively labeled analyte and unlabeled analyte for binding sites on the antibody in the antiserum. By adding to the antiserum known amounts of the analytes to be assayed and a radiolabeled analog, a dose - response curve for bound or free analyte versus concentration of analyte is constructed. After this immunocalibration has been carried out, unknown concentrations can then

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be compared to the standard dose-response curve for assay. Crucial to this type of assay is the existence of radioactive analytes which compete effectively with non-radioactive analytes. Accordingly, in order to obtain the maximum precision, accuracy, sensitivity, specificity and reproducibility of the assay, purified, well-characterized synthetic radioactive analytes are required.

Several deficiencies in radioimmunoassay methodology have been identified. First of all, it is necessary to make a  
10 physical separation of the antibody bound radiolabeled analyte from the free radiolabeled analyte. Further, the methodology is considered rather labor intensive, and the equipment required is likewise relatively expensive, is not uniformly available, and further requires the use of highly trained and skilled technicians to accurately carry out such assays. Likewise, the radioisotopically-labeled analytes are relatively unstable and expensive and pose an increasingly severe waste disposal problem owing to radiation exposure hazards associated with the commonly used radioisotopic labels. Despite these short-  
20 comings, the use of radioimmunoassay has grown considerably.

The substantial recent growth in the use of radioimmunoassay in clinical laboratories has, however, spurred the development of variants which overcome the deficiencies of the radioimmunoassay methodology as described herein. The approaches which have been developed to overcome these deficiencies primarily involve the use of enzyme or fluorescent labels instead of radioisotopic labels, preferably coupled with conditions allowing for measuring a chemical distinction between bound and free fractions of labeled analyte which leads to the  
30 elimination of the requirement for physical separation. Immunoassays having the latter simplifying and advantageous

feature are referred to as homogeneous immunoassays as opposed to heterogeneous immunoassays where physical separation is required.

Thus, homogeneous immunoassay systems have been developed which are based on the use of an enzyme-labeled analyte where the enzymatic activity of the label is decreased when complexation with the antibody occurs. Unlabeled analyte whose concentration is to be determined displaces the enzyme-labeled analyte bound to the antibody, thus causing an increase in enzymatic activity. Standard displacement or dose-response curves are constructed where increased enzymatic activity (monitored spectrophotometrically using what has been termed a "substrate" which ultimately produces a unique chromophore as a consequence of enzyme action) is plotted against increased analyte concentration. These are then used for determining unknown analyte concentrations. The following United States patents have been issued in the field of homogeneous enzyme immunoassay: 3,817,837; 3,852,137; 3,875,011; 3,966,536; 3,905,871; 4,065,354; 4,043,872; 4,040,907; 4,039,385; 4,046,636; 4,067,774; 4,191,613; and 4,171,244. In these patents, the label for the analyte is described as an enzyme having a molecular weight substantially greater than 5,000. Also, commercialization of this technology has been limited so far to applications where the analytes are relatively small in molecular size at fluid concentrations of the analyte greater than  $10^{-10}$  M.

As a consequence of the limitations of the homogeneous enzyme immunoassay technique described above, considerable effort has been devoted towards developing more sensitive homogeneous immunoassays using fluorescence. These have been primarily directed at assays for the larger sized molecules

such as immunoglobulins or polypeptide hormones such as insulin. The following United States patents have been issued for this type of assay: 3,998,943; 3,996,345; 4,174,334; 4,161,515; 4,203,479 and 4,160,016. The label in most of these patents involves an aromatic fluorescent molecule, bound either to the analyte or to the antibody. All likewise involve various methods of quenching fluorescence through antibodies or other fluorescent quenchers so that the extent of quenching is related to the amount of analyte present in the sample.

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A further type of methodology which may be described as a reactant-labeled fluorescent immunoassay involves the use of a fluorescent-labeled analyte designed so that a fluorescent product is released when it is enzymatically hydrolyzed. Antibody to the analyte portion of the molecule, however, inhibits enzymatic hydrolysis. Consequently, by the law of mass action, fluorescence is enhanced in the presence of increased analyte due to enzymatic hydrolysis of the displaced, fluorescent labeled analyte. As an example, a labeled analyte is  $\beta$  - galactosyl-umbelliferone-sisomicin. The enzyme  $\beta$  -galactosidase cleaves the sugar from the umbelliferone moiety which can then fluoresce. Publications which describe this methodology include: J.F. Burd, R.C. Wong, J.E. Feeney, R.J. Carrico and R.C. Boquelaski, Clin. Chem., 23, 1402(1977); Burd, Carrico, M.C. Fetter, et al., Anal. Biochem., 77, 56 (1977) and F. Kohen, Z. Hollander and Boquelaski, Jour. of Steroid Biochem., 11, 161 (1979).

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The co-pending U.S. Farina et al. application, USN 248,689, filed March 30, 1981, provides methodology for carrying out non-isotopic immunoassays which obviates the deficiencies of prior assays

of this general type. In an illustrative embodiment, this methodology utilizes a labeled analyte-polypeptide complex which expresses ribonuclease-type activity to catalytically convert a substrate to a chromogenic or fluorogenic reporter molecule.

Many organic compounds have been utilized heretofore for monitoring the catalytic activity of ribonuclease. Such organic compounds, or substrates, as they are commonly referred to, include ribonucleic acid itself, cyclic phosphate diesters, and monoribonucleotide compounds which exhibit the same or similar structural constraints as those expressed by the natural substrate.

Thus, for example, one method for monitoring the catalytic activity of ribonuclease involves the use of a ribonucleic acid solution. That method involves monitoring a decrease in absorbance at 300 nm of a ribonucleic acid solution as a function of time, M. Kunitz, J. Biol. Chem., 164, 363 (1946). Although that method is relatively simple to conduct, it has several deficiencies; specifically, the rate of decrease of absorption is not linear, calibration of each substrate solution is required, and direct monitoring of absorbance decreases at 300 nm is impractical with clinical samples.

Another method utilized for monitoring ribonuclease activity is an end-point variant of the procedure described above. In the end point variant procedure, yeast ribonucleic acid is incubated with the enzyme sample for a fixed period of time. The remaining RNA is precipitated with perchloric acid or uranyl acetate/trifluoroacetic acid, and the absorbance of the supernatant is measured after centrifugation. S.B. Anfinsen, R.R. Redfield, W.L. Choate, A. Page, and W.R. Carroll, Jour. Biol. Chem., 207, 201 (1954). However, that method is

much too cumbersome for homogeneous immunoassays of the type described in the co-pending Farina et al. application primarily due to the precipitation step involved.

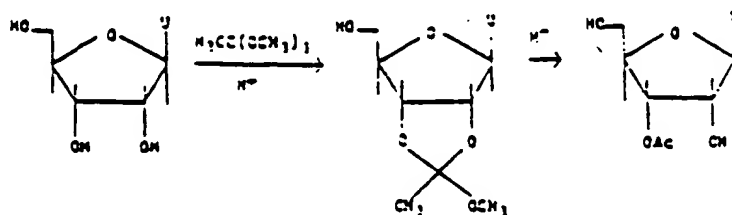
Yet another variation of the above procedures has been reported by R.C. Kamm, A.G. Smith, and H. Lyons, Analvt. Biochem., 37, 333 (1970). The method described therein is based on the formation of a fluorescent reaction product resulting from the reaction of the dye ethidium bromide with intact yeast ribonucleic acid, but not with the hydrolysis products. In that method, a fluorescent signal, which is monitored, decreases with time. However, monitoring a fluorescent signal which decreases with time is disadvantageous, as the method may result in a lack of sensitivity when only modest differences in enzyme concentration are encountered. In addition, other disadvantages are that the rate of decrease of absorption is not linear, and calibration of each substrate solution is required.

Another known substrate for monitoring ribonuclease activity is a mononucleotide substrate, cytidine 2', 3'-phosphate diester, E.M. Crock, A.P. Mathias, and B.R. Rabin, Biochem. J., 74, 234 (1960). In that method, an increase of absorbance at 286 nm, corresponding to the hydrolysis of the cyclic phosphate ring, is monitored over a two-hour period to measure the ribonuclease activity of the sample. This method, however, cannot be used in homogeneous immunoassay methods of the type described in the/above-mentioned Farina et al. co-pending application because there are analyte sample interferences which occur at 236 nm. Furthermore, the distinction between the substrate and product absorbance spectra is small, with the ratio of extinction coefficients being only 1.495 at 286 nm.



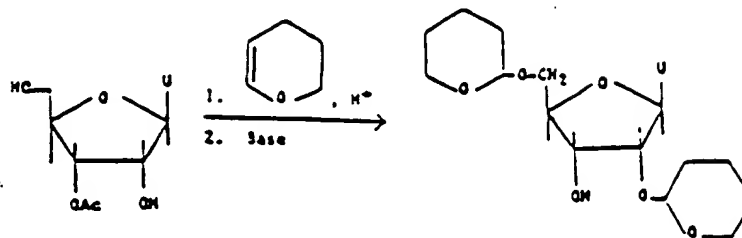
Further, certain mononucleotide-3'-phosphodiester, including, 1-naphthyl esters of 3'-uridylic, 3'-inosonic and 3'-adenylic acids have been utilized as ribonuclease substrates. These naphthyl esters have been used to differentiate substrate specificities of ribonucleases from various sources. R. Sierakowska, M. Zan-Kowalczevska, and D. Shugar, Biochem. Biophys. Res. Comm., 19, 138 (1965); M. Zan-Kowalczevska, A. Sierakowska, and D. Shugar, Acta. Biochem. Polon., 13, 237 (1966); R. Sierakowska and D. Shugar, Acta. Biochem. Polon., 18, 143 (1971); R. Sierakowska, R. Szemplinska, D. Shugar, Biochem. Biophys. Res. Comm., 11, 70 (1963). As a result of ribonuclease-induced hydrolysis, the use of such substances results in the liberation of 1-naphthol which is allowed to react with a diazonium salt to form an azo compound having strong visible absorbance. This approach requires that the assay kit include a separately packaged dye former (viz. - a diazonium salt). Also, this substrate cannot be employed in a fluorometric mode.

Various syntheses have been developed heretofore for the preparation of mononucleotide-3'-phosphodiester. One such method for the preparation of uridine-3'-(1-naphthyl) phosphate is that disclosed in R. Kole and R. Sierakowska, Acta Biochim. Polon., 18, 187 (1971). In accordance with the method shown therein, uridine is acetylated at the 3'-hydroxyl position:

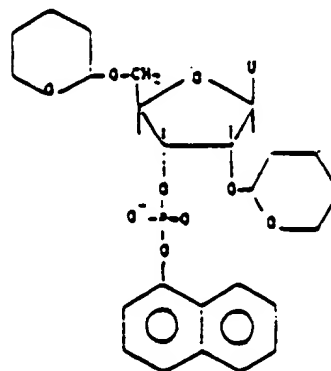


Next the 2'- and 5'-hydroxyl groups of 3'-O-acetyluridine are blocked with dihydropyran; and sequentially, the

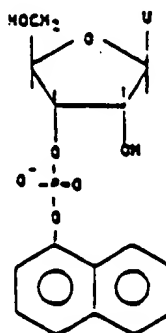
3'-O-acetyl undergoes hydrolysis so that 2', 5'-bis-O-(tetrahydropyranyl) uridine is formed:



Condensation of 2', 5'-bis-O-(tetrahydropyranyl) uridine with naphthyl phosphate/dicyclohexylcarbodiimide or naphthyl phosphoryldichloride then results in 1-naphthyl phosphorylation of the 3'-hydroxyl to form the blocked form of the substrate 2', 5'-di-O-(tetrahydropyranyl) uridine-3'-(1-naphthyl) phosphate:



The tetrahydropyranyl blocking groups are acid labile and may be removed without competitive phosphate hydrolysis to form the substrate, uridine-3'-(1-naphthyl) phosphate:



A variation of the synthesis described in Sierakowska and Shugar discussed above, is the method described in Rubsamen, Khandler and Witzel (Hoppe-Seyler's) Z.Physiol.Chem., 355, 687 (1974). There, 2', 5'-bis-O-(tetrahydropyranyl)-3'-uridine phosphate is prepared by the reaction of dihydropyran with uridine-3'-phosphate. Dephosphorylation of the 2', 5'-bis-O-(tetrahydropyranyl)-3'-uridine phosphate with, for example, phosphatase or lead (II) hydroxide, forms 2', 5'-di-O-(tetrahydropyranyl) uridine. The 3'-hydroxyl of that compound may then be phosphorylated in the fashion disclosed in Sierakowska and Shugar to form the desired mononucleotide-3'-phosphodiester, such as, for example, uridine-3'-(1-naphthyl) phosphate.

The synthesis schemes described by Sierakowska et al., and Rubsamen et al., suffer, however, from several major deficiencies. For example, in each synthesis method, the preparation of the key intermediate, 2', 5'-bis-O-(tetrahydropyranyl) uridine, involves an undesirable, lengthy chromatography. Further, the resulting product is a mixture of diastereomeric pairs in low yields; and this complicates subsequent synthetic steps. Finally, the overall synthesis is labor-intensive.

Closely similar schemes to those of Sierakowska et al. and Rubsamen et al. are disclosed in Polish Patent No. 81969. In one synthesis described therein, uridine 2', 5'-di-O-tetrahydropyrano-3'-(1-naphthyl) phosphate is formed in dicyclohexylcarbodiimide and pyridine by the reaction of a salt of 1-naphthylphosphoric acid, (e.g., the pyridine, aniline, lutidine or tri-n-butylamine salt of the acid) with 2', 5'-di-O-(tetrahydropyranyl)uridine. In another synthesis described therein, uridine 2'-O-tetrahydropyranyl-5'-O-methyl-3'-(1-naphthyl) phosphate is formed in pyridine by the reaction

of a salt of 1-naphthylphosphoric acid and 5'-O-methyl-2'-O-(tetrahydropyranyl)uridine. These schemes likewise suffer from the deficiencies of the Sierakowska et al. and Rubsamen et al. methods.

In addition, methods are known for preparing oligoribonucleotides which incorporate the synthesis of 2', 5'-diblocked nucleotides as intermediates. Thus, in J. Smrč and F. Šorm, Collection Czechoslov. Chem. Commun. 27, 73 (1962), uridylic acid is converted into 5'-O-acetyluridine 2', 3'-cyclic phosphate which, after enzymatic cleavage of the cyclic phosphate by pancreatic ribonuclease, results in 5'-O-acetyluridine 3'-phosphate, which is then transformed into 2'-O-tetrahydropyranyl-5'-O-acetyluridine 3'-phosphate by the reaction with dihydropyran.

In this method, acetylation at the 5'-hydroxyl of the cyclic phosphate is utilized as a synthetic convenience for preparing intermediates in the synthesis of oligoribonucleotides. Deblocking of the 5'-acetyl is ultimately carried out in the formation of the desired oligoribonucleotide.

This, however, does not describe a suitable method for synthesizing a chromogenic and/or fluorogenic mononucleotide-3'-phosphodiester.

Further, in K.K. Ogilvie, S.L. Beaucage, A.L. Schifman, N.Y. Theriault and K.L. Sadana, Can. J. Chem., 56 2763 (1978), 2', 5'-di-*t*-butyldimethylsilyl blocked uridine and adenosine nucleosides are prepared by the reaction of *t*-butyldimethylsilyl chloride in pyridine or imidazole with a uridine or adenosine nucleoside. The resulting silylated nucleosides are then coupled to one another by phosphorylation to form oligonucleotides.

Moreover, insofar as is known, the Smrč et al. and Ogilvie et al. methods have not heretofore been utilized in

preparing such chromogenic and/or fluorogenic mononucleotide 3'-phosphodiester, despite the deficiencies of prior methods.

Thus, despite the considerable number of methods that have been developed and utilized for synthesizing various substrates suitable for use for monitoring enzymatic or catalytic activity, there remains the need for further development which can overcome the various shortcomings of the presently known synthetic methods. None of the synthesis schemes described heretofore are currently being used commercially for the manufacture of mononucleotide-3'-phosphodiester insofar as is known.

It is, accordingly, an object of the present invention to provide a novel method for synthesizing mononucleotide-3'-phosphodiester having a chromogenic and/or fluorogenic functional group at the 3' phosphate moiety <sup>in the furanoside ring/</sup> ~~in a more direct~~ manner involving fewer synthetic steps than required in prior methods.

Another object of this invention is to provide a novel method for synthesizing chromogenic and/or fluorogenic mononucleotide 3'-phosphodiester <sup>/for use in monitoring catalytic or enzymatic activity/</sup> ~~which is less labor intensive~~ than previous syntheses heretofore known.

Yet another object of this invention is to provide a novel synthesis of chromogenic and/or fluorogenic mononucleotide 3'-phosphodiester which results in improved overall yields of desired substrate.

Still another object is to provide a novel synthesis of chromogenic and/or fluorogenic mononucleotide-3'-phosphodiester, which may be carried out on a multigram scale sufficient for commercial use.

A further object of the present invention is to provide a product capable of being stored for extended periods

of time and then, when needed, may be converted to an active form for use.

These and other objects and advantages of the present invention will become apparent from the following detailed description.

While the invention is susceptible to various modifications and alternative forms, there will herein be described in detail the preferred embodiments. It is to be understood, however, that it is not intended to limit the invention to the specific forms disclosed. On the contrary, it is intended to cover all modifications and alternative forms falling within the spirit and scope of the invention as expressed in the appended claims. For example, while the present invention will be primarily described in conjunction with the formation of a uridine-3'-phosphodiester, it should be appreciated that bases other than uracil may be employed, as will be described herein.

#### SUMMARY OF THE INVENTION

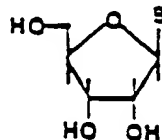
In general, the present invention is predicated on the discovery that mononucleotide-3'-phosphodiester substrates having a chromogenic and/or fluorogenic functional group at the 3'-phosphate moiety may be readily synthesized in as few as three steps by simultaneously blocking the 2'-, 5'-hydroxyls of a mononucleoside with a selected common blocking group and thereafter converting the diblocked species to incorporate the desired chromogenic and/or fluorogenic moiety at the 3'-position. The resulting product is provided in a form capable of being stored for extended periods of time without adverse affects and may then be converted to a useful form by removing the blocking group at least at the 2'-position. The necessary

synthetic steps may be accomplished in a variety of schemes, providing sufficient versatility to allow a synthesis tailored to the desired diester substrate.

The resulting chromogenic and/or fluorogenic mononucleotide-3'-phosphodiester substrates may be utilized for monitoring the catalytic activity of a variety of enzymes, such as, for example, ribonuclease A, T<sub>2</sub>, and the like; and/or polypeptide pairs having the catalytic activity of such enzymes. The chromogenic and/or fluorogenic mononucleotide substrates formed by the method of this invention are useful in the immunoassay methodology disclosed in the previously identified copending Farina et al. application.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with one embodiment of the present invention, suitable starting materials comprise mononucleosides having the following structural formula:



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In this structure, there appear to be certain steric constraints which must be met in order to ultimately provide a substrate suitable for monitoring the catalytic activity of, for example, ribonuclease A-induced hydrolysis. Thus, the trans, cis orientation of the base B and substituents at positions 1'- and 2'-, 3'-, respectively, appear to have rigid structural constraints to provide a suitable substrate. However, the substituents at the 4'-position, that is, CH<sub>2</sub>OH, may apparently have a configuration where the

CH<sub>2</sub>OH group is cis to both the 2'- and 3'- functional groups, without affecting the desirable attributes of the substrate.

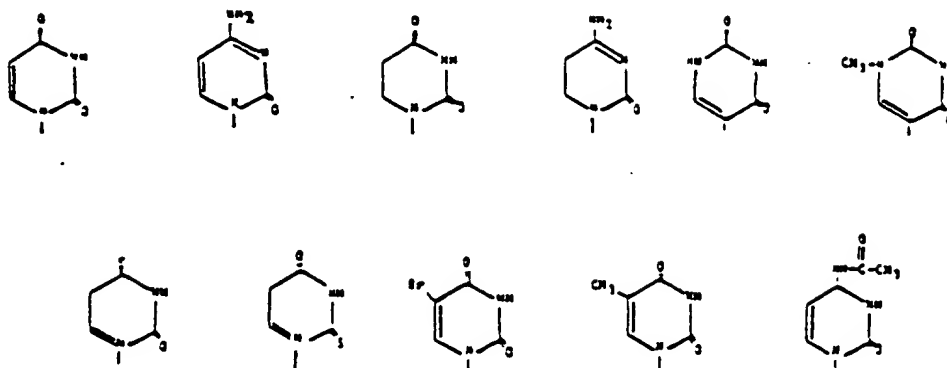
A. Holy and F. Sorn, Biochemica. Biophysica. Acta., 161, 26 (1968). Accordingly, while the method of the present invention will be described in conjunction with the preparation of a substrate wherein the 4'-CH<sub>2</sub>OH substituent is trans to the 2'-, 3'-substituents, it should be appreciated that the method is likewise equally applicable to the preparation of a substrate wherein the 4'-CH<sub>2</sub>OH substituent is cis to the 2'-, 3'-substituents.

From the functional standpoint, the selection of the base should take into account the following factors, in addition to, of course, its effect on product stability: (1) any modulation (increase or decrease) of catalytic activity, (2) the difficulty of synthesis, (3) the effect on endogenous enzymatic activity and (4) the solubility in aqueous or other mediums of interest should not be adversely affected to any significant extent. Other factors to consider include possible effects on hydrolysis and non-specific medium induced hydrolysis.

A wide variety of pyrimidine analogs are useful bases, including uracil, dihydrouracil, cytosine, dihydrocytosine and halogenated uracils. Additionally, based on data extrapolated from results on the ribonuclease-induced hydrolysis of both the natural substrate, RNA, as well as various synthetic substrates, such as, for example, nucleotide homopolymers, F.M. Richards and W.W. Wykoff in The Enzymes, (P.D. Boyer, Ed.), Academic Press, 3d Edition, Volume 4, pages



647-806, London and New York (1978), the following pyrimidine analogs should be suitable bases:

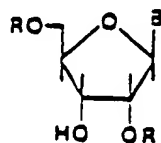


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While the use of purine analogs as bases, such as, for example, adenosine and guanosine, will not provide active substrates for monitoring the catalytic activity of ribonuclease A, these bases should prove useful when ribonuclease T<sub>2</sub> activity is involved. Further, any other pyrimidine, purine or the like analogs may be used consistent with the functional considerations set forth herein.

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In carrying out the first step of the method, the mononucleoside is reacted with a silylating reagent to form a 2'-, 5'-disilylblocked mononucleoside of the general formula:



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Suitable blocking groups R should meet the following criteria: (1) readily introduced without affecting the

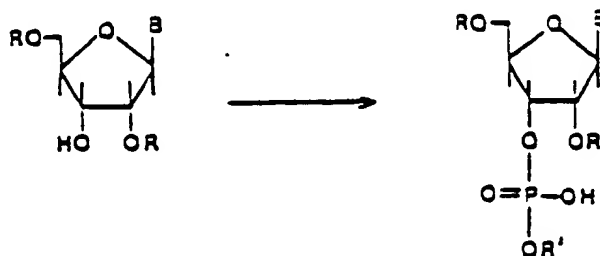
other key functionalities, (2) compatible with the subsequent phosphodiester formation step, and more particularly, should minimize or eliminate undesired side reactions in such step, (3) sufficiently stable to allow long-term storage without any adverse deleterious effects and (4) easily removed without disruption of the phosphodiester bond. These criteria are satisfied by various silyl derivatives including triisopropylsilyl, tert-butyltetramethylenesilyl and tert-butyldimethylsilyl. The tert-butyldimethyl silyl moiety is preferred.

10           In carrying out the silylation step, the resulting products comprise, in addition to the desired 2'-, 5'-diblocked species, a series of isomers including the 3'-, 5'-; 2'-, 3'-, 5'-and 2'-, 3'-diblocked species. The principal isomers provided will typically be the desired 2'-, 5'-diblocked species and the 3'-, 5'-diblocked product. The product mixture obtained will depend upon the particular process parameters employed, and selectivity to the desired species may be enhanced by appropriate selection of such process parameters. Regardless, the separation of the various isomers to  
20       provide the desired diblocked species is relatively simple (e.g. - carried out by straightforward known chromatographic methods) and is not unduly complicated even where selectivity is considerably less than optimum, as might occur when the reaction time is relatively short.

          As one illustrative example, the silylation step may be carried out by using a relative molar ratio of uridine to silylation reagent of about 1:3 to about 1:4 in pyridine, which serves both as a solvent and as a base for catalyzing the reaction. The reaction proceeds satisfactorily at ambient  
30       temperatures and will provide high selectivity and conversion for the desired species after a reaction time of 60 hours or so.

The particular process parameters can be varied within wide limits, as may be desired. A variety of solvents are useful, including dimethylformamide, dimethylsulfoxide, tetrahydrofuran, dioxane and the like. Other useful catalysts include imidazole, 2, 6-lutidine, triethylamine or the like. Useful temperatures range from about 10°C. to about 50°C. with reaction times ranging from no more than 20 minutes or so to as much as 100 hours or so.

The second step of the procedure in accordance with the present invention involves the formation of 2'-, 5'-diblocked  
 10 chromogenic and/or fluorogenic uridine-3'-phosphodiester by the reaction of the 2'-, 5'-diblocked mononucleoside with a suitable derivative form of the chromophore and/or fluorophore, as depicted below:



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The mononucleotide-3'-phosphodiester so formed may be readily handled and chromatographed due to their enhanced solubility in organic solvents, if desired.

Functionally, R' can be defined as any functional group which will provide the substrate with fluorogenic and/or chromogenic properties. The R' group may be an aryl, aralkyl, heteroaryl or heterocyclic compound. In the preferred embodiment, R' is umbelliferonyl, 4-methylumbelliferonyl and 3-flavonyl. Other suitable R' groups include  
 30

aryls such as, for example, 1-naphthyl. Further, other R groups which are suitable are aryl groups which incorporate electron withdrawing and conjugating substituents which increase the acidity of ortho and para benzoic acids. Such groups include, ortho, meta and para nitrophenyl, dinitrophenyl, cyanophenyl, acylphenyl, carboxyphenyl, phenylsulfonate, phenylsulfonyl and phenylsulfoxide. In general, mixtures of mono and bi-substituted derivatives may likewise be suitable.

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The formation of the desired phosphodiester should be conducted, of course, in a fashion adequate to insure that no or only a minimum disruption of the key functionalities occurs. Desirably, the formation should likewise allow use of relatively mild reaction conditions to provide a product capable of being readily isolated in high yields. These general objectives may be suitably accomplished by, in general, either reacting the uridine directly with a phosphorylated derivative of the desired chromophore and/or fluorophore or by first converting uridine to a 3'-phosphorylated derivative and then reaction with an alcoholic derivative of the chromophore and/or fluorophore.

20

The first approach can be carried out by two synthetic schemes. One scheme involves reaction of the phosphate derivative of the chromophore/fluorophore with the 2', 3'-diblocked mononucleoside in the presence of a suitable condensation agent. Importantly, the reagent selected should provide a high yield of ester product at mild reaction conditions. Toluenesulfonyl chloride, mesitylenesulfonyl imidazolide, p-toluenesulfonyl imidazolide, 2, 4, 6-triisopropylbenzenesulfonyl chloride, mesitylenesulfonyl chloride, picrylsulfonyl chloride, N, N'-dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and

30

other carbodiimide analogs with or without additives such as, N-hydroxy-succinimide, and N-hydroxyphthalimide are illustrative examples. The use of 2, 4, 6-triisopropylbenzenesulfonyl chloride and N, N'-dicyclohexylcarbodiimide have been found to be satisfactory.

10       The relative ratio of 2'-, 5'-diblocked mononucleoside to the phosphorylated chromogenic and/or fluorogenic derivative should be in the molar range of at least 1 to 1, desirably employing an excess of such derivative. A ratio of 1:2 has been found satisfactory for the amount of the nucleoside to that of the condensation agent, but excesses up to perhaps 1:5 or so may likewise be perhaps useful.

20       The reaction may be carried out in an aprotic polar solvent such as N, N-dimethylformamide, dioxane or tetrahydrofuran and the like, in the presence of a base such as pyridine, triethylamine and the like. Suitably, dry pyridine base as a solvent may be used at a temperature in the range of from about -20°C. to about 25°C. with a reaction time of 5 to 12 hours. A reaction temperature range of from about -20°C. to about 50°C. and a time period of about 2 to 72 hours should likewise be satisfactory.

30       The second synthetic scheme involves starting with a chromogenic and/or fluorogenic alcohol. The alcohol may first be phosphorylated in situ to form a reactive intermediate which is then reacted with the 2'-, 5'-disilylated blocked mononucleoside to form the phosphodiester. The phosphorylation reaction may be carried out by employing any of the numerous phosphorylation reagents known in the art, such as, for example, phosphorous oxychloride, 2, 2, 2-trichloroethyl phosphorodichloridite, or the like. The phosphorylation is carried out, typically utilizing an excess of the

phosphorylation reagent, in an aprotic solvent such as N, N-dimethylformamide, dioxane, tetrahydrofuran or the like, in the presence of a base, such as, pyridine or triethylamine. Pyridine may be used as the solvent and base. The excess phosphorylating reagent may then be removed from the reactive intermediate before further reaction to form the 2', 5'-disilylblocked mononucleoside phosphodiester.

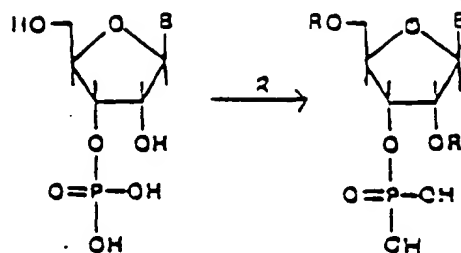
Suitable reaction conditions include employing dry pyridine base at a temperature in the range of from about -10°C. to about 30°C. and a reaction time of about 1 to about 3 hours. A temperature range of about -78°C. to about 80°C. and reaction times of from about 5 minutes to about 5 hours should likewise be suitable.

In a first alternative, but related aspect of the invention, the 2', 5'-disilylated blocked mononucleoside may be phosphorylated to form a diblocked mononucleotide intermediate, which may then be reacted with a chromogenic and/or fluorogenic alcohol to form the 2', 5'-disilylated blocked mononucleotide phosphodiester.

In the second approach, the 2', 5'-diblocked nucleoside derivative is first phosphorylated to form a reactive intermediate which is then reacted with the chromophore and/or fluorophore alcohol. The phosphorylation and subsequent ester forming reactions may suitably be carried out as previously described in conjunction with the first approach. The phosphorylated nucleoside derivative can be either the acid chloride or the acid itself. In the latter instance, a condensation reagent should be employed, as has been previously described in conjunction with the first approach.

In accordance with yet another embodiment of the present invention, a 2', 5'-disilylblocked mononucleotide is

first prepared by the reaction of a 3'-mononucleotide and a silylating reagent, as shown schematically below:



10

wherein R is a silyl blocking group, and B is a base, as previously defined herein. The 2', 5'-disilylblocked uridine monophosphate may then be condensed with the chromogenic and/or fluorogenic alcohol to form the 2', 5'-disilylblocked mononucleotide 3'-phosphodiester. The silylation and condensation reactions may be carried out utilizing the process parameters previously discussed in the first approach.

20

The 2', 5'-disilylblocked substrate is a stable compound which may be stored for extended periods of time. However, deblocking is necessary to provide a suitable substrate for use in monitoring the enzymatic or catalytic activity in applications such as non-isotopic immunoassays.

Deblocking of the substrate may be readily carried out with the use of several different reagents without significant deleterious hydrolytic cleavage of the deblocked substrate.

The reagents should, of course, be mild enough so that the phosphate diester bond is not cleaved during the deblocking step. Also, and importantly, the reagent should be capable of being readily separated from the phosphodiester substrate and should not inhibit or otherwise interfere with the subsequent intended application. Suitable reagents include acids, ammonium halide salts, inorganic halide salts and the like. Generally, tetrabutylammonium fluoride, tributylfluoroborate, lithium tetrafluoroborate, hydrogen fluoride,   
 10 acetic acid, and hydrochloric acid may be used. Tetrabutylammonium fluoride has been found suitable.

The deblocking reaction is generally carried out in a protic or aprotic polar solvent such as tetrahydrofuran, acetonitrile, dioxane, pyridine or water using an excess of the deblocking reagent. As an example, a 1M solution of tetrabutylammonium fluoride in tetrahydrofuran may be employed at a temperature of about 15°C. to about 30°C. for a period of from about 20 minutes to about 50 minutes. The temperature range may suitably extend from about 0°C. to about 50°C. with   
 20 reaction times of as little as about 10 minutes up to perhaps 120 minutes or so.

The following Examples are merely illustrative of the present invention and are not intended as a limitation on the scope thereof.

#### EXAMPLE I

This Example illustrates the preparation of 2', 5'-bis-t-butyldimethylsilyluridine.

In the preparation of 2', 5'-bis-t-butyldimethylsilyluridine, 11.39 g, 0.0466 mole, of uridine was dissolved in   
 30 20 ml of pyridine by stirring at room temperature for about



5 min. Then 21.09 g, 0.140 mole, *t*-butyldimethylsilyl chloride was added to the pyridine solution and the mixture was stirred at room temperature for about 62 hours in a flask fitted with a drying tube. The reaction mixture was diluted with 150 ml ether and then filtered to remove pyridine-HCl. The ether-pyridine filtrate was concentrated on a rotary evaporator and then in high vacuum using a liquid nitrogen trap.

Thin layer chromatography of an aliquot of the  
10 reaction product mixture on silica gel plate, with a solvent of, by volume, two parts ether and one part hexane showed three components, respectively, at  $R_f$  0.65, 0.5 and 0.3.

The remainder of the oily reaction product mixture was chromatographed on a 4.2 x 44 cm silica gel column comprising Silica gel 60 (EM Reagent, Lot No. 7953179), of particle size 0.063 - 0.2 mm and 70 - 230 mesh (ASTM) with a solvent of, by volume, two parts hexane and one part ethyl acetate, to separate the three components of the reaction product mixture. The fractions having  $R_f$  of 0.5, identified by thin  
20 layer chromatography at the conditions given above, were combined. Additionally, fractions containing the  $R_f$  0.3 and 0.65 components were rechromatographed to isolate additional  $R_f$  0.5 product. The  $R_f$  0.5 fractions were combined to give a yield of 3.961 g, that is 40.5%. The melting point (123-125°C.) and n.m.r. spectrum ( $CDCl_3$ ) of the product confirmed the product as 2', 5'-bis-*t*-butylmethylsilyluridine.

#### EXAMPLE II

This Example illustrates the preparation of 2',  
5'-bis-*t*-butyldimethylsilyl-3'-uridine 1-naphthyl phosphate.

30 The disodium salt of 1-naphthyl phosphate, 1.814 g, 6.77 mmoles, was converted into the pyridinium salt using a

Bio-Rad AG<sup>R</sup> 50 W-X8 cation exchange column. The resulting pyridinium salt solution was concentrated at room temperature in vacuum.

10 A solution of 3.197 g, 6.77 mmols of 2', 5'-bis-t-butyldimethylsilyluridine, prepared in Example I, in 50 ml of dry pyridine was added to a solution of the concentrated pyridinium salt in 50 ml dry pyridine. The mixture was dried twice by stripping pyridine off, using 50 ml of dry pyridine for each drying operation. The resulting glassy residue was redissolved in 10 ml of dry pyridine and 2.6 g of 2, 4, 6-triisopropylbenzenesulfonyl chloride was added to the solution. The reaction mixture was stirred in the dark at room temperature for 20 hours and then concentrated to dry-  
ness in vacuo at room temperature.

Thin layer chromatography of a portion of the crude product on silica gel using a solvent system comprised of hexane, methanol, methylene chloride and triethylamine in the ratio, by volume, of 5:2:2:0.5, showed one major spot at  $R_f$  0.5 and minor spots at the origin,  $R_f$  0.81 and  $R_f$  0.75.  
20 Under these conditions, the starting material had an  $R_f$  of 0.75, and the diester had an  $R_f$  of 0.5.

The crude mixture was then chromatographed on a 23 x 2.5 cm Silica Gel G column to separate the components. The column was eluted sequentially with 100 ml of chloroform, 100 ml of 5% methanol in chloroform, and 80% methanol in chloroform until the eluant showed product. Eluant fractions of twenty ml each were collected. Fractions 10 to 13 were identified by thin layer chromatography at the conditions given above, as containing the phosphodiester product ( $R_f$  0.5).  
30 These fractions were combined and concentrated to give 4.721 g of tan colored product. The melting point, 83-86°C.,

and infrared spectrum (in KBr) and n.m.r. spectrum ( $\text{CDCl}_3$ ) of the product confirmed the product as 2', 5'-bis-t-butyldimethylsilyl-3'-uridine 1-naphthyl phosphate.

#### EXAMPLE III

This Example illustrates the preparation of 3'-uridine-(1-naphthyl) phosphate.

10 The 2', 5'-bis-t-butyldimethylsilyl-3'-uridine-(1-naphthyl) phosphate prepared in Example II, 75 mg, was treated with 3.2 ml of 1 M solution of tetrabutylammonium fluoride in tetrahydrofuran. The mixture was stirred at room temperature for 40 minutes, and then the solvent was removed by evaporation in vacuo to leave the crude product residue. The residue was dissolved in 2 ml of water, and extracted 3 times with 5 ml portions each of ether to remove unwanted byproducts. The aqueous solution was blown by a stream of nitrogen to remove any residual ether to thus obtain 3'-uridine-(1-naphthyl) phosphate solution, essentially free of byproducts.

20 The 3'-uridine 1-naphthyl phosphate solution was buffered with 0.1 M sodium acetate buffer of about pH 5 and used in the thyroxine assays set forth in Examples XVII, XVIII and XXIX in the previously identified copending Farina et al. application.

#### EXAMPLE IV

This Example illustrates the preparation of 2', 5'-bis-tert-butyldimethylsilyl-3'-uridine (4-methylumbelliferone-7-yl) phosphate.

30 In this Example, 2', 5'-bis-tert-butyldimethylsilyl-uridine is phosphorylated to form a reactive intermediate which is reacted with 4-methylumbelliferone.

In a round bottom flask, 0.2386 g of 2', 5'-bis-tert-butyldimethylsilyluridine, prepared in Example I, was dissolved in 5 ml of dry pyridine. The solution was evaporated to dryness in vacuo. The solid residue was redissolved in 7 ml of dry tetrahydrofuran and 4 ml of pyridine, and cooled with stirring in an ice-water bath under exclusion of atmospheric moisture. To the stirred cold solution there was added 0.5 ml of phosphorus oxychloride, using an air tight syringe. The mixture was allowed to stir for 5 minutes in a cooling bath, and then at room temperature for 1.5 hours. Pyridine HCl salt was deposited in the bottom of the flask.

An aliquot of the reaction mixture was analyzed by thin layer chromatography to monitor the formation of the intermediate. The chromatography was carried out on a silica gel plate with a solvent system comprising ethyl acetate, chloroform and hexane in the ratio, by volume, of 5:2:3. The analysis showed a component with  $R_f$  near the origin. However, there was no component with  $R_f$  0.55 thereby indicating that the uridine starting material had been completely consumed.

The remainder of the reaction mixture was concentrated in vacuo using a liquid nitrogen trap to remove unreacted phosphorus oxychloride. To the residue there was added 0.107 g of 4-methylumbelliferone, and the mixture was cooled in an ice-water bath under nitrogen atmosphere to exclude atmospheric moisture. To the mixture there was added 4 ml of dry pyridine, and the resulting solution was stirred at room temperature for 40 minutes.

An aliquot of the resulting light yellow solution was analyzed by thin layer chromatography, at the same conditions as given above. A new fluorescent spot, believed to be 2', 5'-bis-tert-butyldimethylsilyl 3'-uridine (4-methylumbelliferone-7-yl) phosphate, was found.

The remainder of the solution was concentrated in vacuo to a glassy oil. The oil was suspended in 5 ml of tetrahydrofuran (THF). To the THF suspension, there was added 20 ml of ether and the mixture was stored in a cold room, about 4 to 8°C., to precipitate product. The product as obtained in this fashion was collected by filtration and dried over  $P_2O_5$  in vacuo to yield 0.572 g of light gray powder. The product was confirmed by n.m.r. to contain 2', 5'-bis-tert-butyldimethylsilyluridine 3'-(4-methylumbelliferone-7-yl) phosphate.

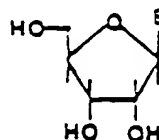
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The 2', 5'-bis-tert-butyldimethylsilyl-uridine-3'-(4-methylumbelliferone-7-yl) phosphate was deblocked following the same procedure as set forth in Example III, to form 3'-uridine-(4-methylumbelliferone-7-yl) phosphate, which was identified by enzyme assay. In an assay with RNase enzyme, the assay mixture was excited at 325 nm and emission was monitored at 450 nm corresponding to the fluorogenic 4-methylumbelliferone, resulting from catalytic hydrolysis of 3'-uridine-(4-methylumbelliferone-7-yl) phosphate.

WHAT IS CLAIMED IS:

1. A method for preparing a mononucleotide-3'-phosphodiester-based substrate capable of undergoing catalytic-induced hydrolysis of the phosphate ester at the 3'-position to yield a species capable of being monitored spectrophotometrically or fluorometrically, comprising

(a) blocking a mononucleoside of the formula:



wherein B is a nucleotide base, and wherein the  $\text{CH}_2\text{OH}$  group at the 4'-position is either cis or trans to said base, with a silyl blocking member at both the 2'-and 5'-hydroxyls of said mononucleoside to form a 2', 5'-diblocked mononucleoside; and

(b) forming a 2', 5'-diblocked mononucleotide 3'-phosphodiester by bonding said 2', 5'-diblocked mononucleoside with a moiety selected from the group consisting of a chromophore and fluorophore;

said silyl blocking member at the 2'-hydroxyl being capable of at least essentially blocking medium-induced hydrolysis of the phosphate ester at the 3'-position, and said silyl blocking member at least at the 2'-hydroxyl being capable of being removed to provide a substrate characterized by the ability to undergo catalytic-induced hydrolysis of the phosphate ester at the 3'-position to yield a species capable of being monitored spectrophotometrically or fluorometrically.

2. The method of claim 1 wherein the silyl blocking member at least at the 2'-hydroxyl is removed so as to provide a substrate characterized by the ability to undergo catalytic-induced hydrolysis of said phosphodiester to yield a species

capable of being monitored spectrophotometrically or fluorometrically.

3. The method of claim 1 wherein said base is a pyrimidine analog.

4. The method of claim 1 wherein said base is a purine analog.

5. The method of claim 1 wherein said base is a member selected from the group consisting of uracil, dimyrouracil, cytosine, dihydrocytosine and halogenated uracils.

6. A method of claim 1 wherein said base is uracil.

7. The method of claim 1, wherein said silyl blocking member is a member selected from the group consisting of triisopropylsilyl, tert-butyltetramethylenesilyl and tert-butyldimethylsilyl.

8. The method of claim 1 wherein said moiety is a member selected from the group consisting of aryl, aralkyl, heteroaryl or heterocyclic compound.

9. The method of claim 8 wherein said moiety is a member selected from the group consisting of umbelliferonyl, 4-methyumbelliferonyl, 3-flavonyl, 1-naphchyl, o-nitrophenyl, m-nitrophenyl, p-nitrophenyl, 2,4-dinitrophenyl, cyanophenyl, acylphenyl, carboxyphenyl, phenylsulfonate phenylsulfonyl and phenylsulfoxide.

10. The method of claim 9 wherein said moiety is 1-naphthyl.

11. The method of claim 9 wherein said moiety is 4-methylumbelliferonyl.

12. The method of claim 9 wherein said moiety is 3-flavonyl.

13. The method of claim 1 wherein said 2', 5'-diblocked mononucleotide 3'-phosphodiester is formed by the reaction of said 2', 5'-diblocked mononucleoside and a phosphorylated derivative of said moiety.

14. The method of claim 13 wherein said reaction is carried out in a condensation reagent selected from the group consisting of toluenesulfonyl chloride, mesitylenesulfonyl imidazolid, p-toluenesulfonyl imidazolid, 2,4,6-triisopropylbenzenesulfonyl chloride, mesitylenesulfonyl chloride, picrylsulfonyl chloride, N, N-dicyclohexylcarbodiimide, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

15. The method of claim 14 wherein said reaction is carried out in the presence of an additive selected from the group consisting of N-hydroxysuccinimide, N-hydroxyphthalimide, 2,4,6-triisopropylbenzenesulfonyl chloride and N,N'-dicyclohexylcarbodiimide.

16. The method of claim 14 wherein the molar ratio of said mononucleoside and said phosphorylated moiety is at least 1 to 1.



17. The method of claim 16 wherein said reaction is carried out in an aprotic polar solvent selected from the group consisting of N,N-dimethylformamide, dioxane and tetrahydrofuran.

18. The method of claim 17 wherein said reaction is carried out in the presence of a base selected from the group consisting of pyridine and triethylamine.

19. The method of claim 18 wherein said base is pyridine.

20. The method of claim 19 wherein said reaction is carried out at a temperature in the range of from about -20°C. to about 25°C.

21. The method of claim 20 wherein said reaction is carried out for a period of from about 5 to about 18 hours.

22. The method of claim 1 wherein said moiety is an alcohol, the alcohol being phosphorylated in situ to form a reactive intermediate, and said reactive intermediate being reacted with said 2',5'-diblocked mononucleoside to form said phosphodiester.

23. The method of claim 1 wherein said 2', 5'-diblocked mononucleoside is phosphorylated to form a 2', 5'-diblocked mononucleotide reactive intermediate, and reacting said mononucleotide reactive intermediate with said moiety to form said phosphodiester.

24. The method of claim 22 or 23, wherein said phosphorylation reaction is carried out in the presence of a phosphorylation reagent selected from the group consisting of phosphorous oxychloride and 2,2,2-trichloroethyl phosphorodichloridite.

25. The method of claim 23 or 24 wherein said phosphorylation is carried out in the presence of an aprotic solvent selected from the group consisting of N,N-dimethylformamide, dioxane and tetrahydrofuran.

26. The method of claim 25 wherein said phosphorylation reaction is carried out in the presence of a base selected from the group consisting of pyridine and triethylamine.

27. The method of claim 26 wherein said phosphorylation reaction is carried out in the presence of pyridine.

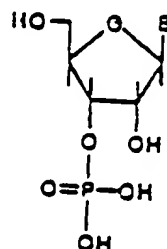
28. The method of claim 27 wherein said phosphorylation reaction is carried out at a temperature in the range of from about -10°C. to about 30°C.

29. The method of claim 28 wherein said phosphorylation reaction is carried out for a time of from about 1 hour to about 3 hours.

30. The method of claim 23 wherein said moiety is an alcohol.

31. A method according to claim 1, comprising

(a) blocking a mononucleotide of the formula:



wherein B is a nucleotide base, and wherein the  $\text{CH}_2\text{OH}$  group at the 4'-position is either cis or trans to said base, with a silyl blocking member at both the 2'- and 5'-hydroxyls of said mononucleotide to form a 2', 5'-diblocked mononucleotide;

(b) forming a 2', 5'-diblocked mononucleotide 3'-phosphodiester by bonding said 2', 5'-diblocked mononucleotide with a moiety selected from the group consisting of a chromophore and fluorophore;

said silyl blocking member at the 2'-hydroxyl being capable of at least essentially blocking medium-induced hydrolysis of the phosphodiester at the 3'-position, and said silyl blocking member at least at the 2'-hydroxyl being capable of being removed to provide a substrate characterized by the ability to undergo catalytic-induced hydrolysis of the phosphate ester at the 3'-position to yield a species capable of being monitored spectrophotometrically or fluorometrically.

32. The method of claim 1 wherein the silyl blocking member at least at the 2'-hydroxyl is removed so as to

provide a substrate characterized by the ability to undergo catalytic-induced hydrolysis of said phosphodiester to yield a species capable of being monitored spectrophotometrically or fluorometrically.

33. The method of claim 2 or 32 wherein said removal of said silyl blocking member is carried out in the presence of a deblocking reagent selected from the group consisting of tetrabutylammonium fluoride, trityltetrafluoroborate, lithium tetrafluoroborate, hydrogen fluoride, acetic acid and hydrochloric acid.

34. The method of claim 33 wherein said removal of said silyl blocking member is carried out in the solvent selected from the group consisting of tetrahydrofuran, acetonitrile, dioxane and water.

35. The method of claim 34 wherein said blocking reagent is tetrabutylammonium fluoride, said solvent is tetrahydrofuran and the concentration of tetrabutylammonium fluoride is 1M in said tetrahydrofuran.

36. The method of claim 35 wherein said reaction is carried out at a temperature of from about 15°C. to about 30°C.

37. The method of claim 36 wherein said reaction is carried out for a period of from about 20 minutes to about 50 minutes.



DOCUMENTS CONSIDERED TO BE RELEVANT															
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)												
X	TETRAHEDRON LETTERS, no.33, Pergamon Press, (GB) K.K. OGILVIE et al.: "The use of silyl groups in protecting the hydroxyl functions of ribonucleosides", pages 2861-2863 * page 2861 *	1-37	C 07 H 19/04 G 01 N 33/48 C 07 H 21/00												
X	--- CHEMICAL ABSTRACTS, vol.90, no.15, April 9, 1979, page 679, abstract no.121925x, Columbus, Ohio (US) K.K. OGILVIE et al.: "The synthesis of oligoribonucleotides. II. The use of silyl protecting groups in nucleoside and nucleotide chemistry, VII." & Can. J. Chem. 1978, 56(21), 2768-80, (Cat. D.) * the whole abstract *	1-37													
Y	--- CHEMICAL ABSTRACTS, vol.96, no.3, January 18, 1982, page 141, abstract no.16415g, Columbus, Ohio (US) D.M. HAWLEY et al.: "Preparation, properties, and uses of two fluorogenic substrates for the detection of 5'-(venom) and 3'-(spleen) nucleotide phosphodiesterases" & Anal. Biochem. 1981, 117(1), 18-23 * the whole abstract *	1-37	C 07 H 19/00 C 07 H 21/00												
The present search report has been drawn up for all claims															
Place of search THE HAGUE		Date of completion of the search 30-06-1982	Examiner VERHULST W.												
<table border="0"><tr><td><b>CATEGORY OF CITED DOCUMENTS</b></td><td><b>T</b> : theory or principle underlying the invention</td></tr><tr><td><b>X</b> : particularly relevant if taken alone</td><td><b>E</b> : earlier patent document, but published on, or after the filing date</td></tr><tr><td><b>Y</b> : particularly relevant if combined with another document of the same category</td><td><b>D</b> : document cited in the application</td></tr><tr><td><b>A</b> : technological background</td><td><b>L</b> : document cited for other reasons</td></tr><tr><td><b>O</b> : non-written disclosure</td><td></td></tr><tr><td><b>P</b> : intermediate document</td><td><b>&amp;</b> : member of the same patent family, corresponding document</td></tr></table>				<b>CATEGORY OF CITED DOCUMENTS</b>	<b>T</b> : theory or principle underlying the invention	<b>X</b> : particularly relevant if taken alone	<b>E</b> : earlier patent document, but published on, or after the filing date	<b>Y</b> : particularly relevant if combined with another document of the same category	<b>D</b> : document cited in the application	<b>A</b> : technological background	<b>L</b> : document cited for other reasons	<b>O</b> : non-written disclosure		<b>P</b> : intermediate document	<b>&amp;</b> : member of the same patent family, corresponding document
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